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Nanoparticle-Decorated Surfaces for the Study of Cell-Protein-Substrate Interactions

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ABSTRACT

The present study was motivated by the need for accurately-controlled and well-characterized novel biomaterial formulations for the study of cell-protein-material interactions. For this purpose, the current research has focused on the design, fabrication and characterization of model native oxide-coated silicon surfaces decorated with silica nanoparticles of select sizes, and has examined the adhesion of osteoblasts and fibroblasts on these nanoparticle-decorated surfaces. The results demonstrate the capability to deposit nanoparticles of select diameters and substrate surface coverage onto native silicon oxide-coated silicon, the firm attachment of these nanoparticles to the underlying native silicon oxide, and that nanoparticle size and coverage modulate adhesion of osteoblasts and fibroblasts to these substrates. The material formulations tested provide a well-controlled and well-characterized set of model substrates needed to study the effects of nanoscale features on the functions of cells that are critical to the clinical fate of implantable biomaterials.

INTRODUCTION

Previous studies have shown that the presence and size of nanoscale features on a material surface affect protein interactions [1] and thus subsequently modulate the functions of different cell types in specific manners [1,2]. For example, osteoblast adhesion is selectively enhanced on nanophase alumina, titania, and hydroxyapatite while fibroblast adhesion is suppressed on these materials [3]. However, definitive studies on the influences of size and distribution of nanoscale surface features on cell functions have not yet been conducted. In particular, the effects of nanoscale feature size and distribution on the type, amount, and conformation of biologically adhesive proteins adsorbing to a surface have not yet been systematically studied. The present work has begun to address these issues. Surfaces decorated with silica nanoparticles of specific sizes were fabricated, substrate coverage by nanoparticles was quantified, and subsequently the stability of particle attachment to the underlying substrate was assessed. Finally, nanoparticle-decorated surfaces were utilized as substrates to investigate the adhesion of osteoblast and fibroblasts.

MATERIALS AND METHODS

Substrates

Native oxide-coated silicon (Silicon Quest Interantional, Inc.) pieces (10 mm square) were degreased and cleaned by soaking and sonicating in acetone for 10 minutes each, rinsing three

times in deionized water, soaking and sonicating in 70% ethanol (Aaper) for 10 minutes each, rinsing three times in deionized water, and finally drying at 120°C for at least 12 hours.

Silica nanoparticle dispersions

Spherical silica particles (Eka Chemicals, Inc.) of 4, 20, or 100 nm average diameter dispersed in water were frozen at -80°C and then freeze-dried for 48-72 hours using a Virtis Benchtop 3.3 freeze dryer to remove water. Stock dispersions of 20.0 mg/ml for the 4 nm, 20.0 mg/ml for the 20 nm, and 5.0 mg/ml for the 100 nm diameter particles in 200 proof ethanol (Aaper) were prepared using the freeze-dried nanoparticles. Stock solutions were dispersed by sonicating in a Fisher Scientific FS60 ultrasonicator for 8 hours, and allowed to settle for 48 hours. The stock dispersions were diluted with ethanol and subsequently spin-coated onto substrate surfaces.

Spin-coating of nanoparticles onto substrates

Nanoparticles were spin-coated onto degreased and cleaned native oxide-coated silicon substrates using a Specialty Coating Systems P6700 spin-coater. Dispersions were applied (aliquots of 50 microliters per application) to native oxide-coated silicon substrates being spun at 2,500 revolutions per minute and heated to 73°C. Each substrate was spun and heated for approximately 17 seconds, to ensure thorough and uniform heating before the first nanoparticle dispersion application. For each nanoparticle diameter and each dispersion concentration, separate substrates received 1 to 10 dispersion applications to achieve a range of substrate surface coverage.

Characterization of nanoparticle-decorated surfaces

Scanning electron microscopy (SEM) utilized either a JEOL JSM-840 SEM (operating at 25 kV) or a JEOL JSM-6335F field emission SEM (operating at 5 kV) for the generation of digital images and the assessment of native oxide-coated silicon surface coverage by nanoparticles. In preparation for SEM, nanoparticle-decorated substrates were sputter-coated with either gold, platinum, or gold/palladium alloy. Uniformity of surface coverage and the presence of any particle agglomeration were qualitatively assessed from examination of micrographs (1,500X magnification) of representative substrate areas.

The area of each substrate covered by nanoparticles was determined quantitatively from high resolution SEM images (taken at 80,000X for 4 nm particles, 30,000X for 20 nm particles, and 10,000X for 100 nm particles) with ImageJ software (Dr. Wayne Rasband, National Institutes of Health, USA), using the ImageJ Particle Analyzer tool. Averaging data from four high magnification SEM images of a characteristic nanoparticle-decorated surface yielded the surface coverage for that sample. The average surface coverage for each discrete combination of particle size, dispersion concentration, and number of dispersion applications was measured using values of samples from three separate preparations.

Nanoparticle-decorated surface submersion tests

To investigate the possible release of silica nanoparticles from the nanoparticle-decorated native oxide-coated silicon, substrates were submersed in cell culture media and kept under standard cell culture conditions (a static, humidified, 37°C, 5% CO_2/95% air environment), without media replacement for 3, 5, and 7 days. After the prescribed time, an aliquot (500 microliters) of supernatant media from each submersed sample was deposited onto an individual carbon tape-coated aluminum SEM stub and dried in an oven at 120°C. Energy dispersive X-ray spectroscopy (EDS) was performed within the JSM-840 SEM operating at 25 kV.

Cell cultures

The present study used osteoblasts from neonatal rat calvariae, isolated and characterized according to standard procedures [4], at passage number 2-5. Rat skin fibroblasts purchased from, and characterized by, the American Type Culture Collection (ATCC; cell line CRL-1213) were also used at passage number 25-30.

Osteoblasts and fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (P/S, Gibco) under standard cell culture conditions. Replacement of supernatant cell culture media with fresh media occurred every other day.

Cell adhesion

Either osteoblasts or fibroblasts were seeded (3,500 cells/cm^2) onto the nanoparticle-decorated substrates of interest to the present study and allowed to adhere under standard cell culture conditions for 4 hours. At the end of the prescribed time period, gentle rinsing with phosphate buffered saline removed non-adherent cells. Adherent cells were fixed *in situ* with 10% buffered formalin for 15 minutes. The nuclei of these fixed mononuclear cells were fluorescently stained with Hoechst (Sigma) and counted using fluorescence microscopy. The number of nuclei in five representative fields per substrate was counted, averaged, and normalized using counts of cells adhering on respective native oxide-coated silicon substrates (control). Each cell adhesion experiment was run in duplicate and repeated at two separate times.

RESULTS AND DISCUSSION

Figure 1 shows representative high magnification SEM images of native oxide-coated silicon surfaces decorated with 100 nm silica particles. Quantitative analysis of such images established that the application techniques developed and used in the present study provided control of the subsequent surface coverage by nanoparticles ranging from 0% (no applications, native oxide-coated silicon) to 100% (complete coverage by particles) for 100 nm diameter particles. Figure 2 illustrates the quantitative values of native oxide-coated silicon surface coverage by 100 nm diameter particles obtained from different stock dispersion dilutions and number of dispersion applications to native oxide-coated silicon substrates. Similar results were obtained for 20 nm and 4 nm diameter nanoparticles spin-coated onto native oxide-coated silicon substrates using the same methodology. Specifically, surface coverages up to 100% were

Figure 1. High magnification SEM images of native oxide-coated silicon substrates decorated with 100 nm silica nanoparticles at (A) 13%, (B) 45%, and (C) 82% surface coverage. Each scale bar = 1 μm.

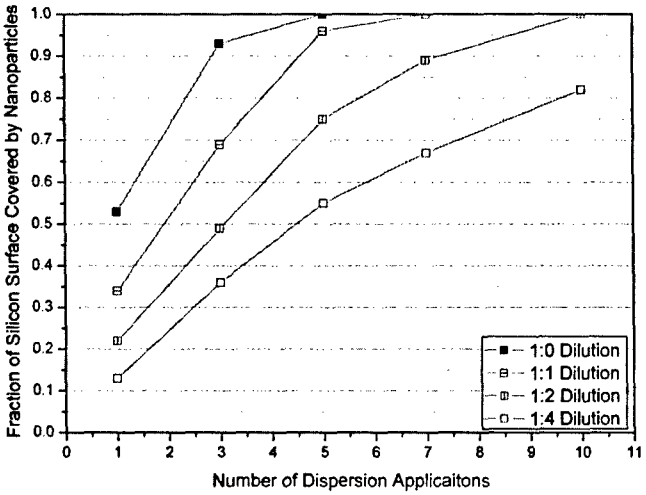

Figure 2. Fraction of native oxide-coated silicon substrate surfaces coverage by 100 nm particles as a function of stock dispersion dilution and number of dispersion applications.

achieved using 20 nm particles, but a maximum of approximately 80% was achieved with 4 nm silica particles. Inability to achieve coverage over 80% using 4 nm particles was attributed to the high tendency of these small particles to agglomerate.

Figure 3 shows several representative EDS spectra of dried supernatant cell culture media (DMEM supplemented with 10% FBS and 1% P/S) from nanoparticle-decorated surface submersion tests. These results revealed no detectable silicon (characteristic X-ray energy of

1.74 keV) in the supernatant media regardless of the silica particle size or duration (up to 7 days) of substrate submersion. This evidence demonstrated that silica nanoparticles did not spontaneously detach from the underlying native oxide-coated silicon surface and did not disperse in the supernatant media. Therefore, changes in cell behavior cannot be attributed to the presence of nanoparticles within the supernatant media; changes in cell adhesion therefore, can only result from the influence of the underlying nanoparticle-decorated surfaces.

Adhesion of osteoblasts and fibroblasts to native oxide-coated silicon and native oxide-coated silicon decorated with 4 nm particles at 20% coverage, 20 nm at 25% coverage, and 100 nm particles at 100% coverage is shown in Figure 4. Osteoblast adhesion was significantly reduced on native oxide-coated silicon decorated with 100 nm silica particles compared to undecorated native oxide-coated silicon and surfaces decorated with either 20 nm or 4 nm particles at the coverages tested. Osteoblast adhesion to 4 nm silica nanoparticle-decorated surfaces was significantly reduced compared to 20 nm silica particle-decorated surfaces and undecorated native oxide-coated silicon (control). Fibroblast adhesion was significantly reduced on native oxide-coated silicon surfaces decorated with either 20 nm particles or 4 nm particles compared to native oxide-coated silicon decorated with 100 nm particles and undecorated native oxide-coated silicon (control). In addition, fibroblast adhesion to 100 nm silica decorated native oxide-coated silicon surfaces was significantly reduced compared to undecorated native oxide-coated silicon (control). The adhesion results of both osteoblasts and fibroblasts onto the substrates of interest to the current study are in agreement with previously reported results for the same cell types adhering to similarly structured, powder-consolidated materials [3].

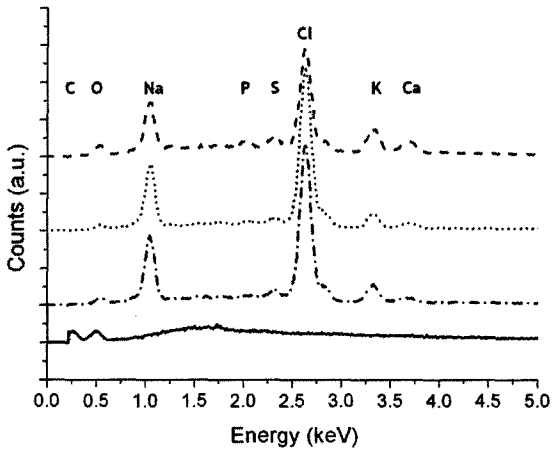

Figure 3. Representative EDS Spectra for supernatant media from 4 nm particle-decorated surfaces (20% coverage, 7 days; dash trace, — — —), supernatant media from 20 nm particle-decorated surfaces (25% coverage, 5 days; dot trace, ------), supernatant media from 100 nm particle-decorated surfaces (100% coverage, 3 days; dash-dot trace, —·—·—), and carbon tape (solid trace, ————). Detected elements are designated by their elemental symbols above the corresponding approximate peak positions. There was no detectable signal for silicon in the EDS spectra of any dried supernatant media samples tested.

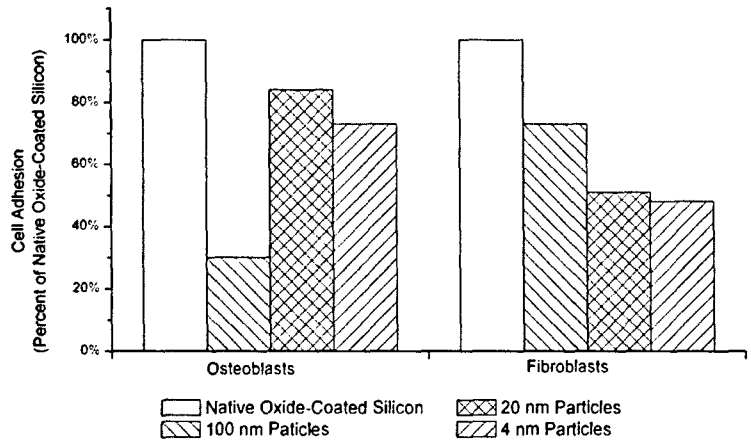

Figure 4. Osteoblast and fibroblast adhesion to undecorated native oxide-coated silicon and native oxide-coated silicon substrates decorated with 4 nm (20% coverage), 20 nm particles (25% coverage), and 100 nm (100% coverage) particles. Values are normalized to adhesion of the respective cell type on undecorated native oxide-coated silicon (control). Data are average of two separate experiments each run with duplicate substrates.

The materials formulated and fabricated for the present study provide a well controlled set of substrates needed for a systematic study of the effects of nanoscale features on the functions of different cells that are critical to the clinical fate of biomedical implant devices. Evidence supporting this goal was provided by control of surface coverage of native oxide-coated silicon substrates by different size nanoparticles, stability of particle attachment to native oxide-coated silicon substrates, and *in vitro* adhesion of mammalian cells (osteoblasts and fibroblasts) on the nanoparticle-decorated surfaces tested.

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